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DEPENDENCE OF MEASURED FLUORESCENCE SIGNAL ON FLUOROPHORE CONCENTRATION

by

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OBJECTIVE

The objective of this work is to develop a model which predicts the major factors affecting the fluorescence signal detected by a commercial fluorescence probe. The probe considered has a surface-type light-source/detector configuration and utilizes the excitation and emission wavelengths corresponding to the fluorescence of NAD(P)H, which is a fluorophore found in all living material. From this model, the relationship between the fluorescence detected and the concentration of fluorescent species can be derived. Using this relationship, the probe can be used to estimate on-line the biomass concentration or the level of NAD(P)H within the cells during a fermentation. The model will also predict how other factors will influence the fluorescence signal.

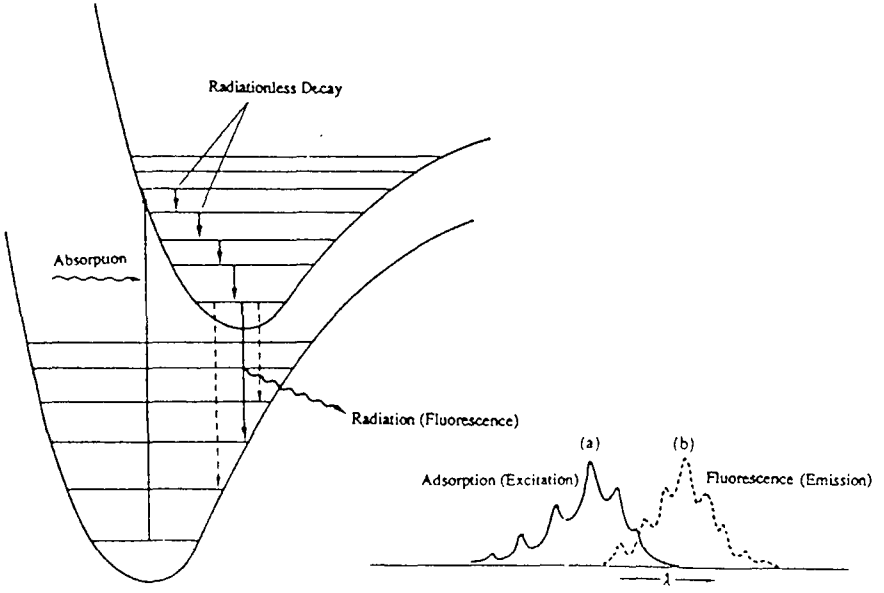
INTRODUCTION

A. Fluorescence as an analytical tool:

Advantages of fluorescence:

- Instantaneous response.
- Nondestructive.
- Wide range of application–Many compounds are fluorescent.
- Selective–Due to differences in both the emission and absorption spectra, interference due to multiple sources of fluorescence can often be limited by choosing appropriate wavelengths for the excitation and detected emission light.

Why do molecules fluoresce?:



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Some biological fluorophores:

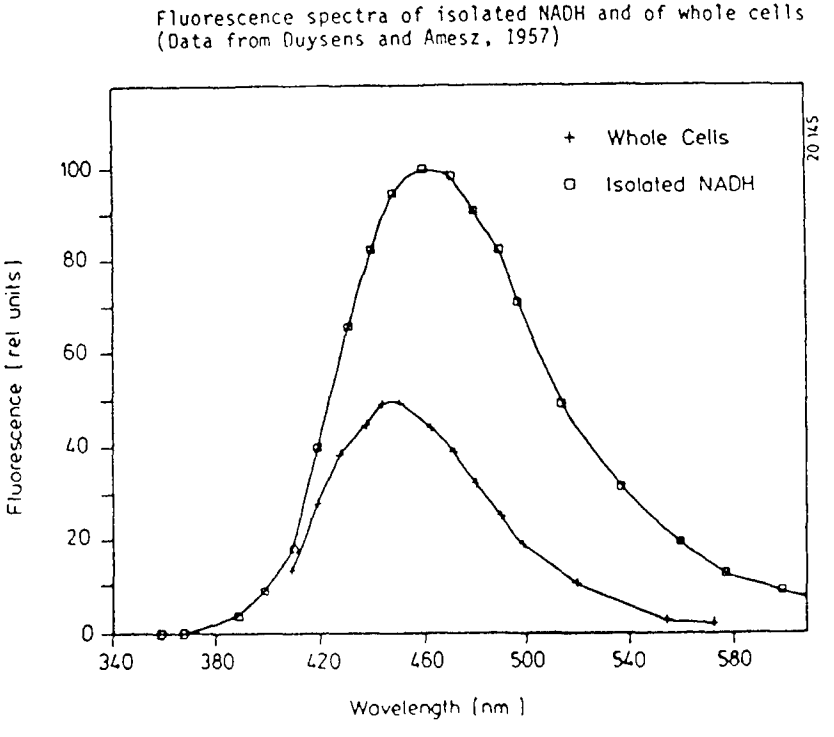
	Fluorophore	*Excitation Wavelength* (nm)	*Emission Wavelenth* (nm)
Electron Carriers	NAD(P)H (reduced form only)	340	460
	F_{420} (oxidized form only)	425	472
	Riboflavins	445	520
	Flavins	450	535
Amino Acids	Tyrosine	275	303
	Tryptophan	287	348
	Phenylalanine	260	282
	Histamine	340	480
	p-Aminobenzoic acid	294	345
	Vitamin B_{12}	275	305
	Estrogens	285	325
	ATD, ADP, etc	272	380
	Serotonin	295	330
	L-Dopa	345	410
	Vitamin A	372	510
	Antibiotics	Var.	Var.

B. NAD(P)H fluorescence probe used in fermentations

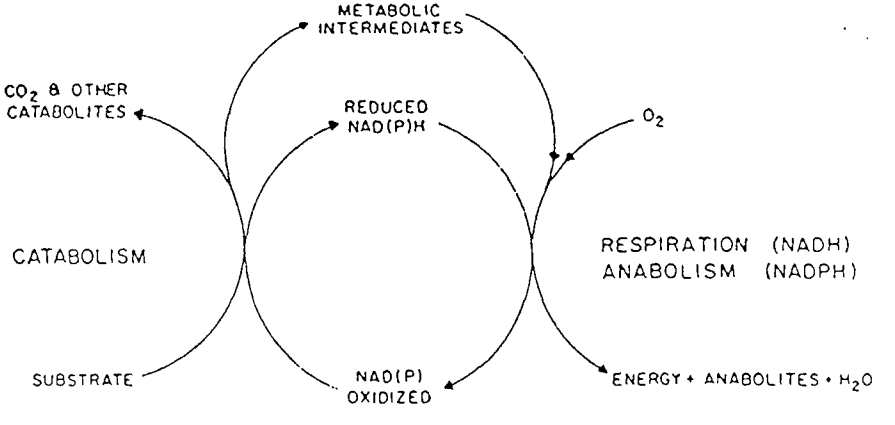
Why NAD(P)H fluorescence can be a valuable measurement:

- NAD(P)H is found in all living material–Dead cells soon lose their NAD(P)H. Thus, the measurement distinguishes living and dead cells.
- The NADH fluorescence contributes up to 60% of the total cellular fluorescence detected by the probe.
- NADH is central to a cells metabolism. The oxidized form, NAD^+, is not fluorescent. Major metabolic changes will influence the NADH level in the cells, and thus, affect the fluorescence signal.

Comparison of yeast and NADH fluorescence:



Role of NADH in central metabolism:



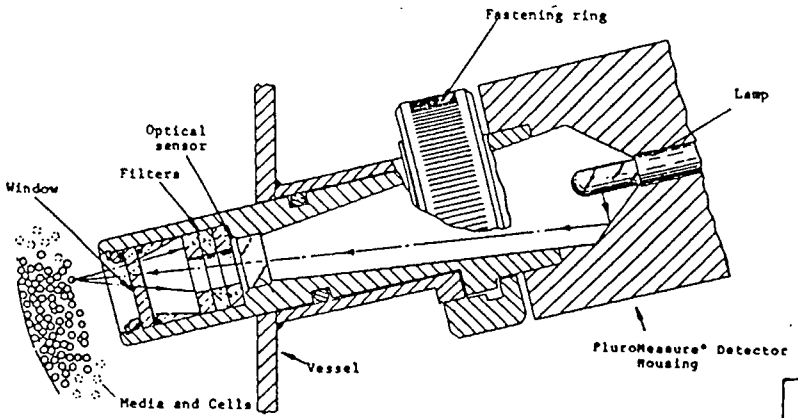
Oxidation-reduction cycle of pyridine nucleotides.

• From Armiger, W.B. *et al.*, *Chem. Eng. Commun.* 45, 197, 1986.

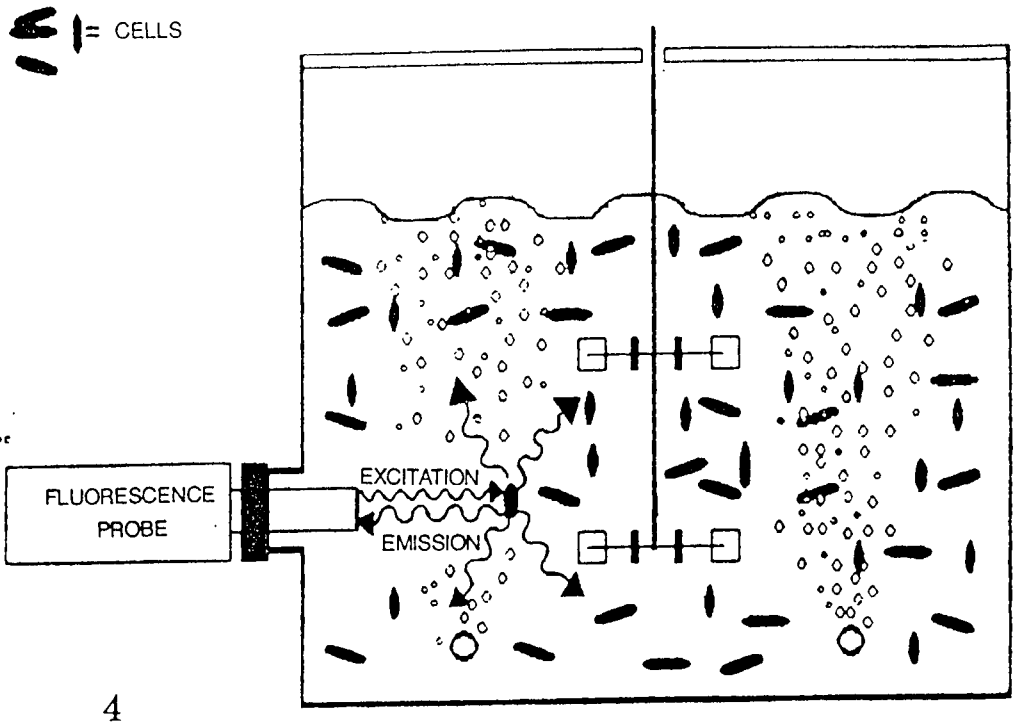
Schematic of the NADH probe fitted into a fermentor

Two producers of this type of probe–Ingold, BioChemTechnology, Inc.

A schematic of the BioChem Technology FluroMeasure* Detector inserted into a culture vessel.



* From Armiger, W.B. *et al.*, paper presented at *British Laboratory Week*, Olympia, London, UK, 1986.



C. What has the NADH fluorescence probe been used for?:

Applications

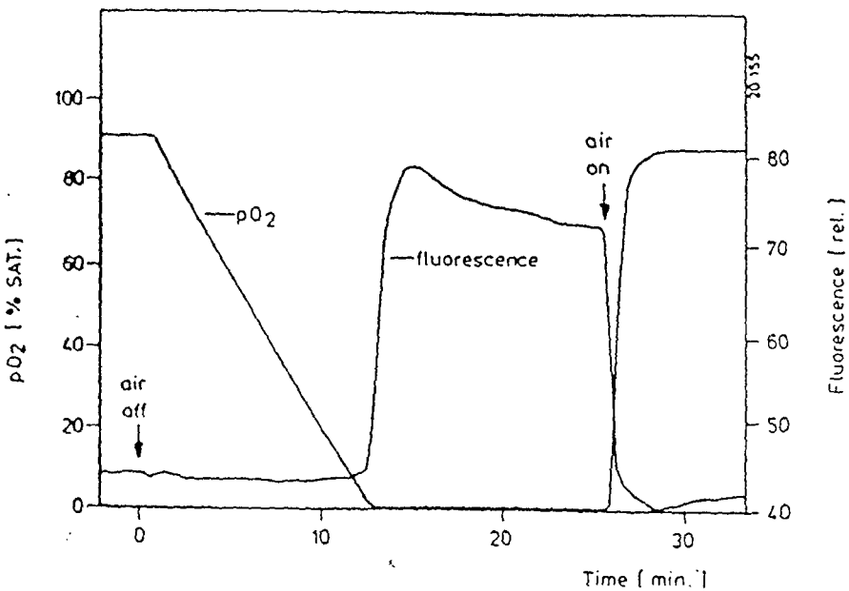
1. Determination of critical dissolved oxygen level.
2. Control of aeration or agitation to avoid anaerobiosis.
3. Measurement of substrate mixing, uptake, and utilization.
4. Recognition of metabolic transitions–substrate depletion, change of substrate, change from anaerobiosis to aerobiosis, product formation, etc.
5. Substrate feed-rate control to avoid glucose repression.
6. Biomass estimation.

2–5 are based on the redox state of NAD(H).

Examples

1. Determination of critical pO_2 level.

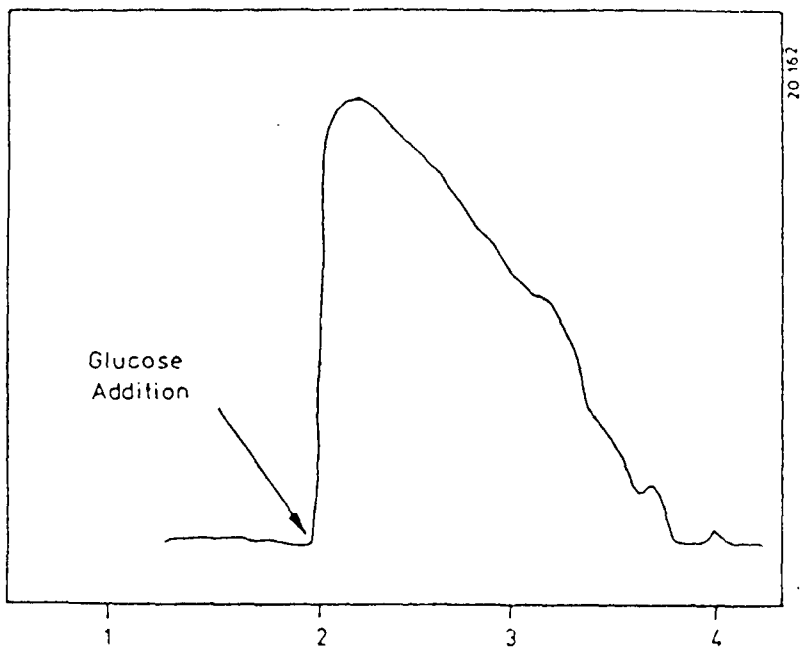
NADH-dependent fluorescence and partial pressure of a continuous culture of Candida tropicalis during aerobic-anearobic transitions. (Data from Beyeler 1981.)



Examples

3. Measurement of substrate mixing, uptake, and utilization.

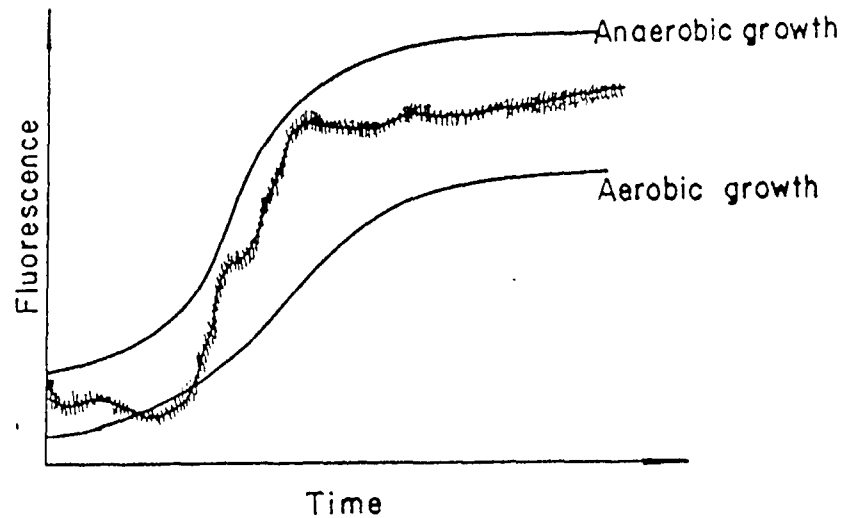
Fluorescence signal after addition of glucose to substrate limited cells. (Data from Scheper 1985)



Examples

5. Substrate feed-rate control to avoid glucose repression.

Substrate Feed Rate Control in Production of Baker's Yeast



Examples

6. Biomass estimation.

Organisms Used for Biomass Estimation by Fluorescence

Baker's Yeast
Bacillus subtilis
B. amyloliquefaciens
Candida tropicalis
E. coli
Methylmonas mucosa
Clostridium acetobutylicum
Pediococcus sp.
Penicillium chrysogenum
* methanogenic bacteria
Streptomyces sp.
Zymonmonas mobilis
Mouse fibroblast
mixed lymphocytes (B&T)
Hybridoma

* Based on F_{420} fluorescence.

Correlations Used for Biomass Estimation

$$X = K\Delta F \qquad \text{(linear)}$$

$$X = a + b\Delta F + c(\Delta F)^2 \qquad \text{(parabolic)}$$

$$X = a'\Delta F^{b'} \qquad \text{(log-log)}$$

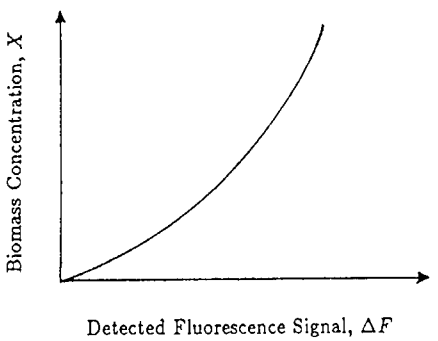
$$X = \alpha \exp(\beta \Delta F) \qquad \text{(semi-log)}$$

$$X = K'(\Delta F \cdot 10^{0.5(\mathrm{OD}_\lambda + \mathrm{OD}_{\lambda'})}) \qquad \text{(abs. corrected (}\perp\text{))}$$

$$X = K''(\Delta F \cdot (\mathrm{OD}_\lambda + \mathrm{OD}_{\lambda'} + \mathrm{S})) \qquad \text{(abs. corr. this work)}$$

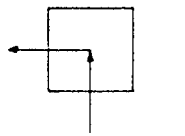
$$X = \frac{\Delta F}{\alpha' - \Delta F \beta'} \qquad \text{(this work)}$$

Typical Fluorescence Correlation

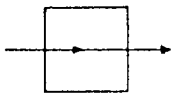


D. Problems–Motivation for this study:

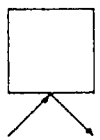
- Fluorescence signal is not a linear function of the fluorophore concentration.



(a) Right-Angle Detector

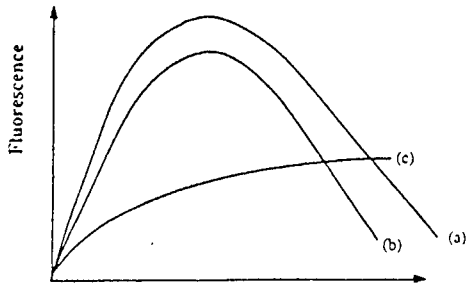


(b) End-On Detector



(c) Surface Detector

Fluorescence Response in Various Configurations



- Fluorescence signal is influenced by many other factors beside the fluorophore concentration.

Factors Affecting Fluorescence Signal

1. Biomass concentration
2. Redox state at the cells: NADH/NAD
3. Environmental factors: pH, T, cell size, aeration rate, medium color or turbidity, background fluorescence
4. Geometric configuration

Why applications listed in section C have worked:

* Applications listed in section C have succeeded because either the applications depend on changes in the fluorescence signal or the shape of the fluorescence curve as plotted against time *or* the factors that affect the signal were kept constant during the experiment.
* Most experiments were ran in or near the linear region of the fluorescence-fluorophore relationship.

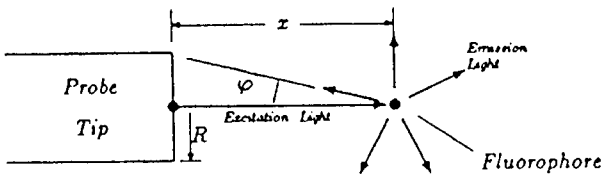
MODEL DEVELOPMENT

A. Comparison of classical analysis and that of this study

Back-scatter configuration – *Assumptions*

Classical analysis and extension to multiple components	This study
1. Beer-Lambert absorption of the excitation light.	1. Beer-Lambert absorption of the excitation light.
2. Amount of detectable fluorescence emitted is proportional to the amount of excitation light absorbed.	2. Amount of detectable fluorescence emitted is proportional to the amount of excitation light absorbed.
3. Negligible absorption of emitted light.	3. Emitted light absorbed according to the Beer-Lambert law. The path length traveled by the emitted light from fluorophore to detector is assumed to be equal to that traveled by the excitation light from the light source to the fluorophore.
4. Nature of light-source is unimportant.	4. Light-source is a point source that puts out a parallel beam. (Divergent beam case to be considered in the near future.)
5. No light-scattering or fluorescence resulting from absorption of emitted light.	5. Presently, only one reflection is considered. Soon, the model will consider up to two reflections.
6. Constant "monitoring efficiency" for all locations in the sample. (A constant fraction of the total fluorescent light is detected.)	6. The fraction of emitted light that is aimed at the detector is given by: $f = \frac{1}{2}(1 - \frac{x}{\sqrt{x^2+R^2}})$ and approximated with $f \sim \frac{1}{2}\exp(-xS)$, where x is the path length and R is the detector radius.

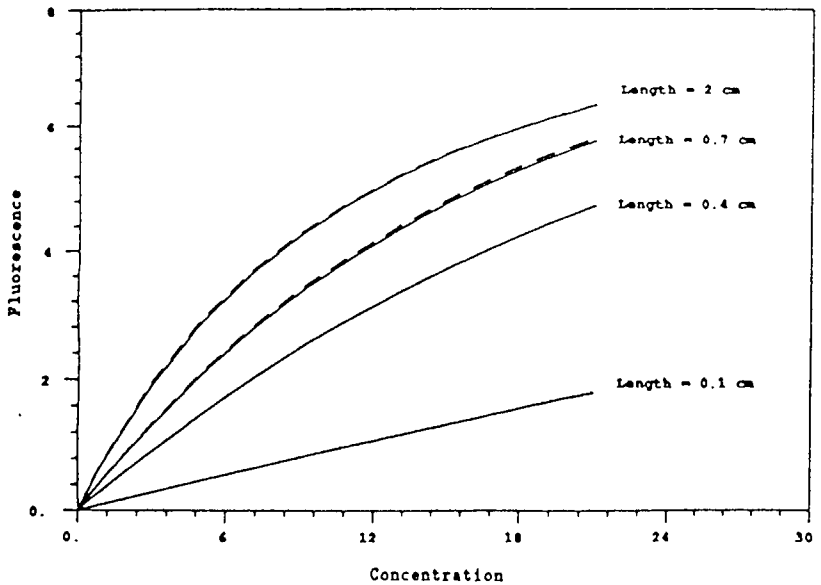
The fraction of the total light emitted that is aimed back at the detector surface is defined by the cone angle, φ, as shown in the figure. Expressed in terms of the distance from the probe, x, and the radius, R, of the effective detector surface, this fraction is given by: $f = \frac{1}{2}(1 - \frac{x}{\sqrt{x^2 + R^2}})$. This is approximated by $f \sim \frac{1}{2}\exp(-xS)$. The effect of this approximation is shown in the graph below.



The effect of approximating the factor, $f(x,R) = (1 - \frac{x}{\sqrt{x^2 + R^2}})$ with $f(x,S) \sim e^{-xS}$. Without making the approximation, the fluorescence is predicted to be:

$$\Delta F = I_\lambda(0)\phi_{\lambda\to\lambda'}a_\lambda c \int_0^L \left\{1 - \frac{x}{\sqrt{x^2 + R^2}}\right\} \exp\left[-x(a_\lambda + a'_\lambda)c\right] dx.$$

Below is a comparison of the result obtained by numerically integrating this with equation B1, shown below, using $I_\lambda(0) = 200$; $\phi_{\lambda\to\lambda'} = 0.0556$; $a_\lambda = 0.09$; $a_{\lambda'} = 0.01$; $R = 1.0\,\mathrm{cm}^{-1}$; and $S = 1/R = 1.2\,\mathrm{cm}^{-1}$. The integration is plotted as the dashed line. Equation B1 is plotted as the solid line.



Notation

$a_{i,\lambda}$; $a_{i,\lambda'}$ Exctinction coefficient of species i at the excitation and emission wavelegths, respectively.

c_i; c Concentration of species i.

F Measured fluorescence intensity.

$I_\lambda(0)$ Intensity of light-source as it enters the sample solution.

L Total path length in the sample.

N Number of species in a multi-component mixture.

OD_λ; OD'_λ Absorbance of the sample at the excitation and emission wavelengths, respectively.

R Effective radius of the detector surface.

S Geometric parameter used to approximate the monitoring efficiency as a function of the position in the sample.

λ; λ' Wavelengths of the excitation and detected emission light, respectively.

$\phi_{i,\lambda\to\lambda'}$; $\phi_{\lambda\to\lambda'}$ Fluorescent yield – Ratio of the amount of the light emitted at wavelegths detectable by the probe, around λ', to the amount of light absorbed at λ. Differs from the "quantum yield", because only the emitted light at wavelegth, λ' is considered in this case.

φ Cone angle which defines the monitoring efficiency at a any position in the sample.

Back scatter configuration – *Results*

Classical analysis and extension to multiple components

A1. Single Component:

$$\Delta F = I_\lambda(0)\phi_{\lambda\to\lambda'}\left[1-\exp(-a_\lambda cL)\right]$$

A2. Multiple Components:

$$\Delta F = \frac{I_\lambda(0)\sum_{i=1}^{N}\phi_{i,\lambda\to\lambda'}a_{i,\lambda}c_i}{\sum_{i=1}^{N}a_{i,\lambda}c_i}\left[1-e^{-L\sum_{i=1}^{N}a_{i,\lambda}c_i}\right]$$

A3. Single Component, Infinite Path Length:*

$$\Delta F = I_\lambda(0)\phi_{\lambda\to\lambda'}$$

* Note–Predicts no concentration dependence.

A4. Absorbance Correction:

$$\Delta F = \frac{1-\exp[-L\cdot \mathrm{OD}_\lambda]}{\mathrm{OD}_\lambda} I_\lambda(0)\sum_{i=1}^{N}\phi_{i,\lambda\to\lambda'}a_{i,\lambda}c_i$$

This study

B1. Single Component:

$$\Delta F = \frac{I_\lambda(0)\phi_{\lambda\to\lambda'}a_\lambda c}{(a_\lambda+a'_\lambda)c+S}\left[1-\exp\left(-L[(a_\lambda+a'_\lambda)c+S]\right)\right]$$

B2. Multiple Components:

$$\Delta F = \frac{I_\lambda(0)\sum_{i=1}^{N}(\phi_{i,\lambda\to\lambda'}a_{i,\lambda}c_i)\left[1-e^{-L\left[\sum_{i=1}^{N}(a_{i,\lambda}+a_{i,\lambda'})c_i+S\right]}\right]}{\sum_{i=1}^{N}(a_{i,\lambda}+a_{i,\lambda'})c_i+S}$$

B3. Single Component, Infinite Path Length:

$$\Delta F = \frac{I_\lambda(0)\phi_{\lambda\to\lambda'}a_\lambda c}{(a_\lambda+a'_\lambda)c_i+S}$$

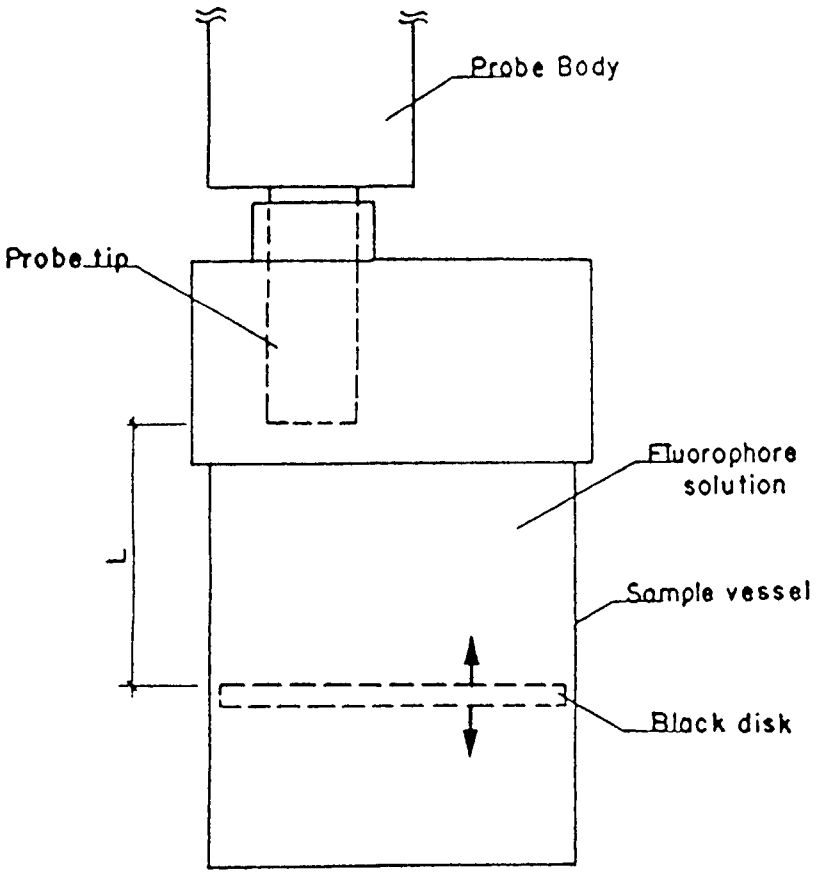
B4. Absorbance Correction:

$$\Delta F = \frac{1-\exp[-L(\mathrm{OD}_\lambda+\mathrm{OD}'_\lambda+S]}{\mathrm{OD}_\lambda+\mathrm{OD}'_\lambda+S} I_\lambda(0)\sum_{i=1}^{N}\phi_{i,\lambda\to\lambda'}a_{i,\lambda}c_i$$

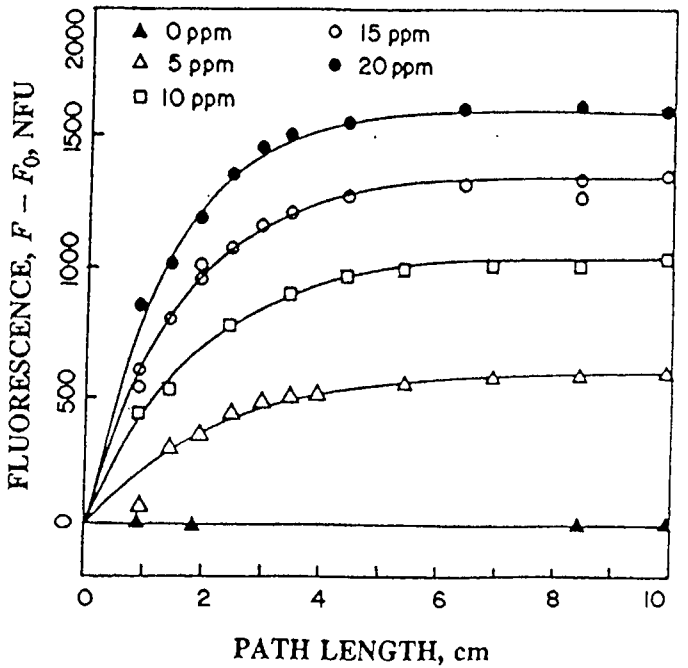
TESTING OF MODEL

Application of Correlational Form to Single-Species Fluorescence–Thioflavin S

- **Apparatus used:**



- **Exponential correlation of constant concentration fluorescence with the path length:**



Correlation suggested by equation B1:

$$\Delta F = F_{max}[1 - \exp(-kL)]$$

where:

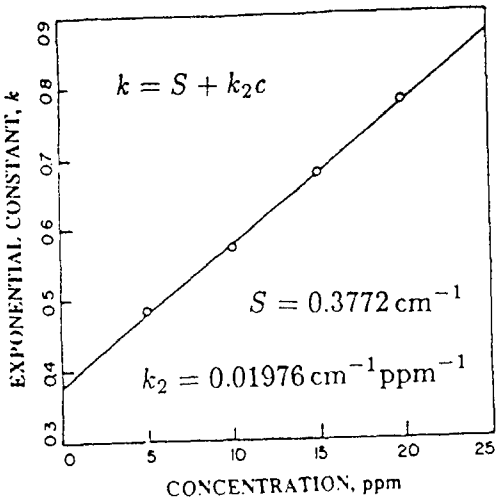
$$F_{max} = I_\lambda(0)\phi_{\lambda\to\lambda'}a_\lambda$$
$$k = (a_\lambda + a'_\lambda)c + S$$

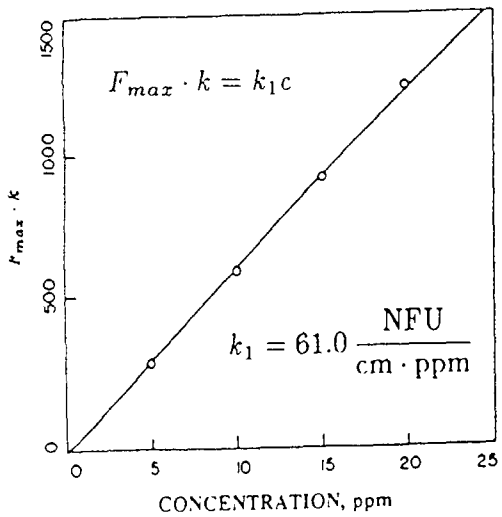
Curves drawn above were fitted using this form with the parameters on the right.

Parameters obtained by fitting experimental fluorescence data:

c	F_{max}	k
5 ppm	605.1	0.4794
10 ppm	1040.1	0.5704
15 ppm	1343.1	0.6720
20 ppm	1595.2	0.7748

- **Correlation of exponential constants with the fluorophore concentration:**





Equation B1 predicts the exponential constants to correlate with the concentration as follows:

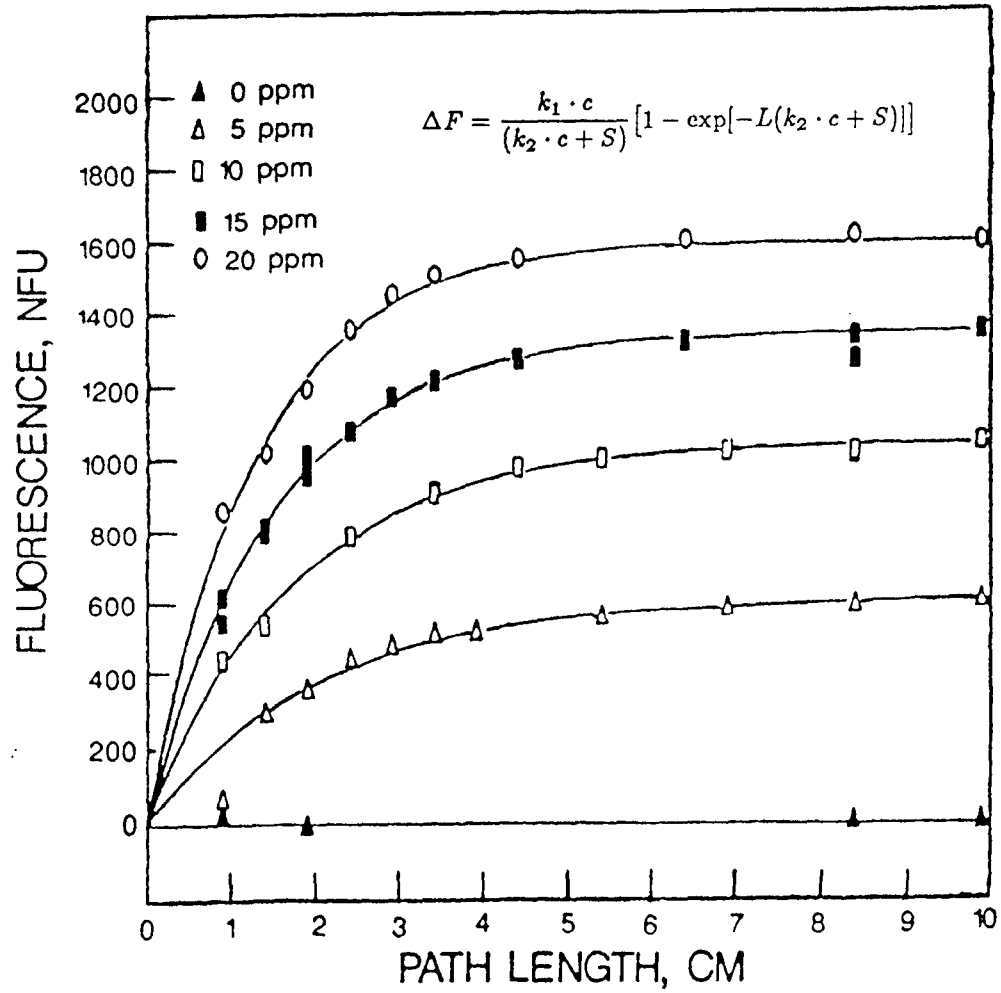
$$F_{max} = \frac{k_1 \cdot c}{(k_2 \cdot c + S)}$$
$$k = k_2 \cdot cS$$

where:

$$k_1 = I_\lambda(0)\phi_{\lambda \to \lambda'} a_\lambda$$
$$k_2 = a_\lambda + a'_\lambda.$$

Note that $F_{max} = \frac{k_1 \cdot c}{k}$. The overall correlation is shown in the graph on the right.

- **Overall correlation with concentration and path length:**

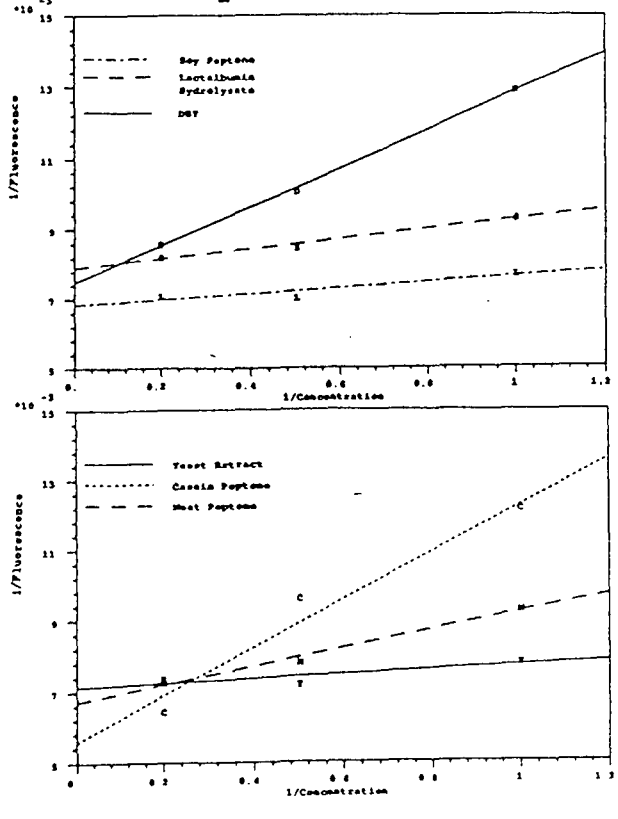


Correlation of fluorescence in common fluorescent media

Complex components can be considered as one species by using "composite" extinction coefficient and fluorescent yield:

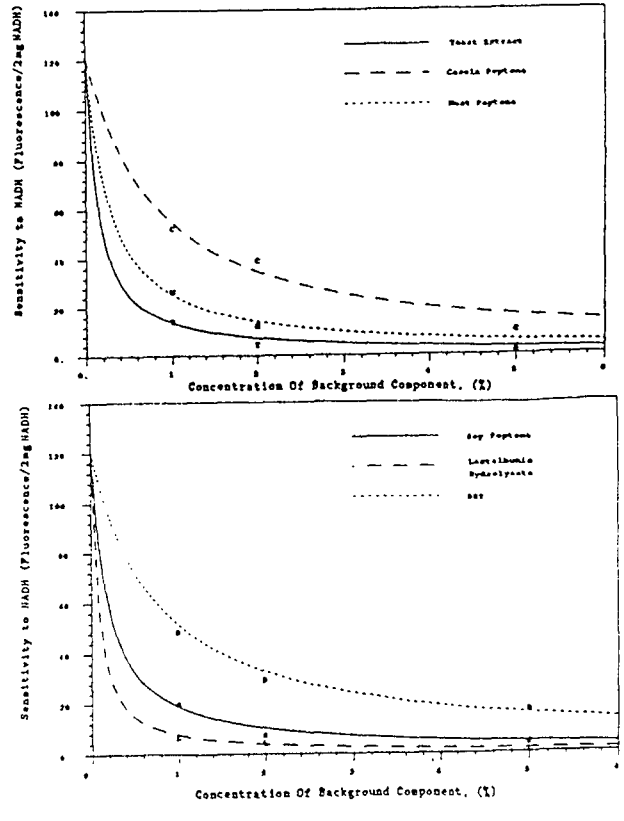
$$a_{(composite)} = \frac{\sum_{i=1}^{N} a_i c_i}{c} \quad \text{and} \quad \phi_{(composite)} = \frac{\sum_{i=1}^{N} \phi_i a_i c_i}{a_{(composite)} c}$$

- **Correlation of the fluorescence of the medium components:**



Fluorescence, ΔF, correlated with concentration, c, according to equation B3.

- **Correlation of the sensitivity to NADH in common fluorescent media**



$\left(\frac{\partial \Delta F}{\partial c_1}\right)_{c_1=0}$ correlated with c_2 according to equation B2 with $L \to \infty$.

* Data obtained from "FluroFacts," BioChemTechnology, Inc.

CONCLUSIONS & RECOMMENDATIONS

- The form of the simple model presented here can be used to correlate the fluorescence of a one- or two-component solution with the fluorophore concentrations.
- The fluorophore-fluorescence relationship is inherently nonlinear. This is primarily due to inner-filtering and geometric effects.
- The classical analysis is not appropriate for an open-ended sample because the "monitoring efficiency" is a function of the position in the sample.
- Inner-filter effects and geometric effects are accounted for in the model.
- Concentration terms are additive, so that composite properties can be used. Also, deconvolution of multicomponent fluorescence is easier.
- The geometric parameters in the model are very important.
- Parameter estimation needs to be improved, possibly by broadening the model to consider divergent light-source, light-scattering, re-emitted light, and non-monochromatic light-source and detector.
- By measuring the sample's absorbance at both the excitation and emission wavelengths, an absorbance correction can be used to linearize the relationship between fluorophore concentration and fluorescence.
- Only one fluorophore concentration can vary, when using only one excitation and emission wavelength pair. When more than one vary, deconvolution is required. Time-decay data as well as the fluorescence at other wavelength pairs can be used for deconvolution of the signal.